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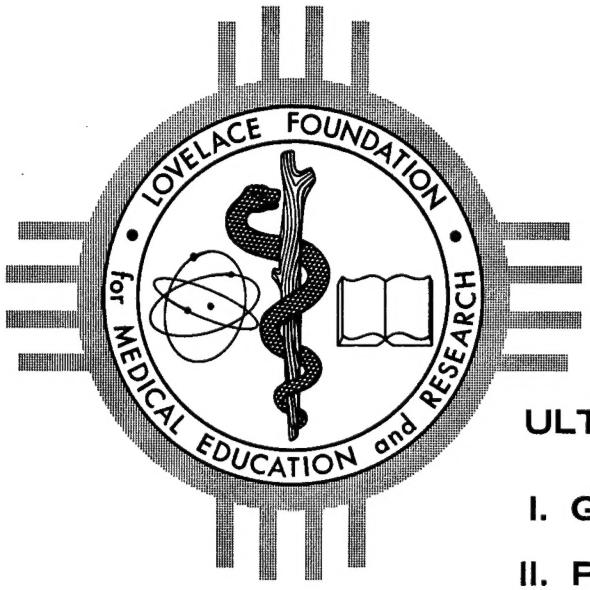

Albuquerque, New Mexico

ULTRAMICRO METHODS IN BIOCHEMISTRY

I. GENERAL CONSIDERATIONS

II. PROCEDURES FOR THE DETERMINATION OF SERUM BILIRUBIN

by

E. VAN STEWART, CHARLES R. PUCKETT
AND AGNES WOOD

October, 1962

ATOMIC ENERGY COMMISSION-
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E. Van Stewart, Charles R. Puckett and Agnes Wood

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ABSTRACT

A battery of biochemical tests to survey the function of body organs and systems of animals under a variety of experimental circumstances was selected. The choice of procedures was guided by an analytical concept, often employed clinically, to reveal early failure of the integrative mechanisms responsible for maintaining homeostasis. More specifically, the methods were chosen to assess general metabolism; to reveal general tissue destruction; and to provide selected data concerning adrenal, thyroid and liver function as well as the status of liver, kidney and bone. The need for serial sampling necessitated the development of an ultramicro system to allow numerous determinations on a small sample of blood. The Sanz ultramicro system was evaluated and various aspects of the associated instrumentation are described and considered.

Ultramicro procedures for determining serum bilirubin utilizing both the diazo reaction and spectrophotometric analysis were investigated. The preparation of a bilirubin standard in aqueous protein solution was developed and evaluated for stability and reproducibility. The relevant diazo coupling and spectrophotometric procedures are described and results comparing the two analytical principles are recorded. Various necessary precautions applicable to these methods are set forth and the advantages and disadvantages of each are detailed and discussed.

ACKNOWLEDGMENTS

The authors are indebted to Dr. Bernard B. Longwell and Dr. Clayton S. White for advice in preparing the manuscript. Too, the assistance of Mr. Robert Smith and Mr. George Bevil, Department of Medical Illustration, is acknowledged as is the secretarial and editorial aid of Mrs. Beth Brown, Mrs. Ruth Lloyd and Mrs. Maureen Gilmore.

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I. GENERAL CONSIDERATIONS

INTRODUCTION

In determining the biological consequences of exposure to one or more experimental variables, including various types of ionizing radiation, it is often desirable to employ periodic biochemical observations conceived to reveal the functional status of the organs and physiological systems of the body, particularly if malfunction is likely to lead either to lethality or serious disease. Planning a comprehensive approach requires recognition of at least two important essentialities; namely, selection of the spectrum of biochemical procedures most likely to be useful and employment of methods chosen to minimize the factor of repetitive blood loss.

The purpose of this paper is first to present a positive, but initial, approach that has been taken to meet these requirements, and second to report in detail the ultramicro method developed and evaluated for determining serum bilirubin. Subsequent publications will set forth the techniques finalized for other biochemical constituents.

CHOICE OF PROCEDURES

1. Selection of Biochemical Tests

One helpful course in selecting biochemical procedures, under circumstances where it is not known with certainty which specific tests would best reveal early failure of the integrative mechanisms whereby the body maintains local or total homeostasis, is to utilize a battery of tests chosen to be reliable indicators of an altered or diseased state of the several organs or systems of the body. Such a course is likely to increase the probabilities of detecting a deranged metabolism or a functional maladjustment that might well be critical to the animal. Accordingly, particular tests were chosen to reveal information about general metabolism; the function of the liver, kidney, and lung; the thyroid and the adrenal glands; bone metabolism and general tissue destruction.

The specific determinations selected are listed in the first column of Table 1, grouped according to the categories noted above. In total, their employment, within certain limits, can be expected to screen the functional state of an experimentally "stressed" animal. The data obtained may or may not reveal a departure from normal conditions, but in either eventuality, they are likely to be useful. Should the biochemical tests yield essentially normal values, but other measures of biological effect prove positive and suggest a high probability of a biochemically detectable lesion, then an appropriate revision of the procedures used would be indicated. Similarly, if positive biological indicators were uncovered through employment of the initial test battery, use of a more specific biochemical approach to probe the etiological mechanisms at play could be planned and utilized.

2. Sample Size

Since the employment of ultramicro methods seemed to be the only practical means of maximizing the number of biochemical procedures to be accomplished without producing undue stress due to periodic blood loss, the sample volume required for each of the selected biochemical tests was assessed. The possibilities appeared favorable and during early exploratory work, ultramicro methods were selected which met two criteria; i.e., the method must be reliable and at the same time, sample size must be minimal.

Subsequently, activities were initiated to establish and evaluate suitable methods. The second column of Table 1 shows the choice of general methodology that was forthcoming and the third column sets forth the sample volume for each procedure as far as it is established at the present time. The seven question marks indicate that the minimal sample size is not known with certainty, but the figure noted gives the likely volume.

It is significant that all the procedures on blood can be accomplished on 2.5 ml of whole blood, a volume that, even if taken weekly from large experimental animals, could hardly introduce undesired effects. Also, this volume of blood can easily be obtained from small animals on a serial-sacrifice basis as well as serially from certain small animals without undue stress if the sample interval is sufficiently long.

Table 1

List of Determinations, Tissue or Organ Evaluated and Quantity of Blood Required

Determination	General methodology	Quantity of fluid required for one determination
A. General metabolism		
1. Glucose	Enzymatic, photometric	5 µl of whole blood
2. Cholesterol	Photometric	10 µl of serum
3 & 4. Total lipid and phospholipid	Photometric	40 µl of serum
B. Liver function		
5. Bilirubin, total	Photometric	20 µl of serum
6. Bilirubin, free or direct	Photometric	20 µl of serum
7. Total protein	Photometric	5 µl of serum
8. Protein electrophoresis	Electrophoresis	10 µl of serum
9. Alkaline phosphatase	Photometric	20 µl of serum
10. Serum glutamic oxalacetic transaminase	Spectrophotometric	20 µl of serum?
11. Serum glutamic pyruvic transaminase	Spectrophotometric	20 µl of serum?
C. Kidney and lung		
12. Oxygen content and saturation	Spectrophotometric or Gasometric	40 µl of whole blood?
13. Standard CO_2, pH, total CO_2, pCO_2, excess acid, or base	Ultramicro Astrup	140 µl of whole blood
14. Sodium, potassium	Flame photometry	50 µl of serum
15. Chloride	Chloridometer	10 µl of serum
16. Urea nitrogen	Enzymatic, photometric	5 µl of serum
17. Creatinine	Photometric	40 µl of serum
18. Uric acid	Photometric	20 µl of serum
D. Bone		
19. Phosphorus	Photometric	20 µl of serum
20. Calcium Alkaline phosphatase	Titrametric	20 µl of serum
E. Thyroid		
21. Butanol extractable iodine	Spectrophotometric	30 µl of serum?
F. Adrenal		
22. Ketosteroids	Photometric	0.5 ml urine?
23. Ketogenic steroids	Photometric	0.5 ml urine?
G. General tissue destruction		
24. Lactic dehydrogenase	Spectrophotometric	20 µl of serum?

BASIC EQUIPMENT

1. Ultramicro Pipettes

The basic equipment for the ultramicro analyses is that first described by Sanz (1). The system is based upon the use of ultramicro pipettes made of non-wettable polyethylene which may be procured with accurate quantitative dimensions. The reliability of the pipette has been confirmed by Westover et al. (2). The pipettes employed* in developing the methods selected are illustrated in Figures 1 and 2. Measurements are based upon a ratio relationship wherein standards, which are run with every determination, are pipetted with the same pipette as in the unknown sample being subjected to analysis.

After careful training and practice with the pipettes, a very reliable analytical system has been achieved. However, cleanliness is paramount; for the pipettes, even though they are non-wettable, are subject to erratic performance unless kept scrupulously clean. The importance of this fact in ultramicro analysis cannot be overemphasized because a small error in pipetting, either a sample or a standard, may represent a relatively greater percentage of the total volume involved than is the case with macro equipment.

2. Reaction Containers

The reactions, in general, are carried out in ultramicro polyethylene centrifuge tubes having a volume of 400 µl. They are a standard part of the Sanz-Spinco analytical system. In certain instances the ultramicro centrifuge tubes are not satisfactory reaction vessels and more conventional containers of larger size are used. However, after ultramicro measurements of the sample and standards, the final accurate total volume can be any convenient quantity designed to produce instrumental readings within the accepted limits.

3. Colorimetric and Spectrophotometric Instruments

Final analyses in methods utilizing reactions involving color or requiring spectrophotometric techniques are dependent upon the availability

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*Purchased from the Spinco Division of Beckman Instruments, Inc., Palo Alto, California.

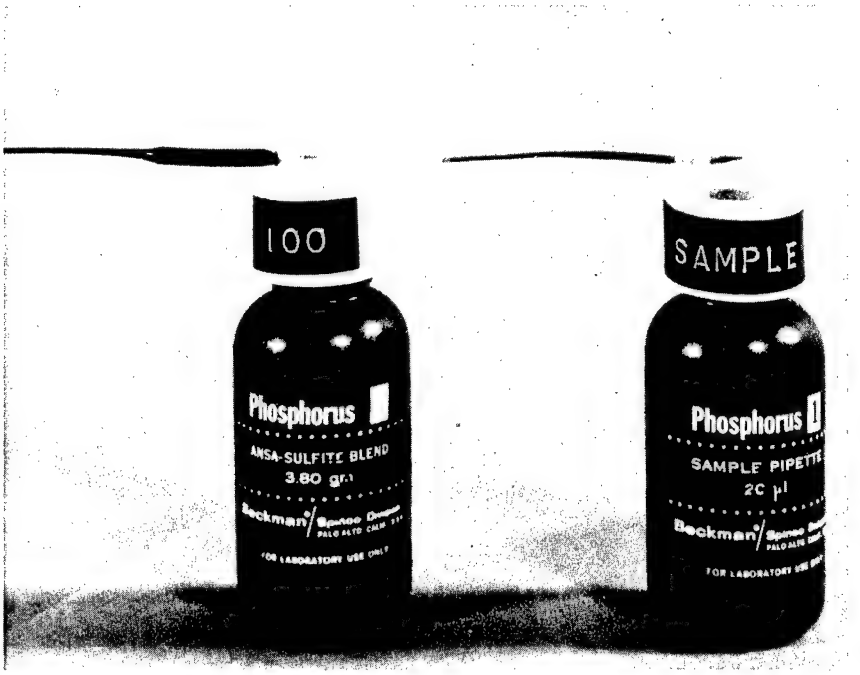

Figure 1. Examples of the Sanz pipette as manufactured by Beckman/Spinco Division. A reagent (left) and a sample (right) pipette are illustrated.

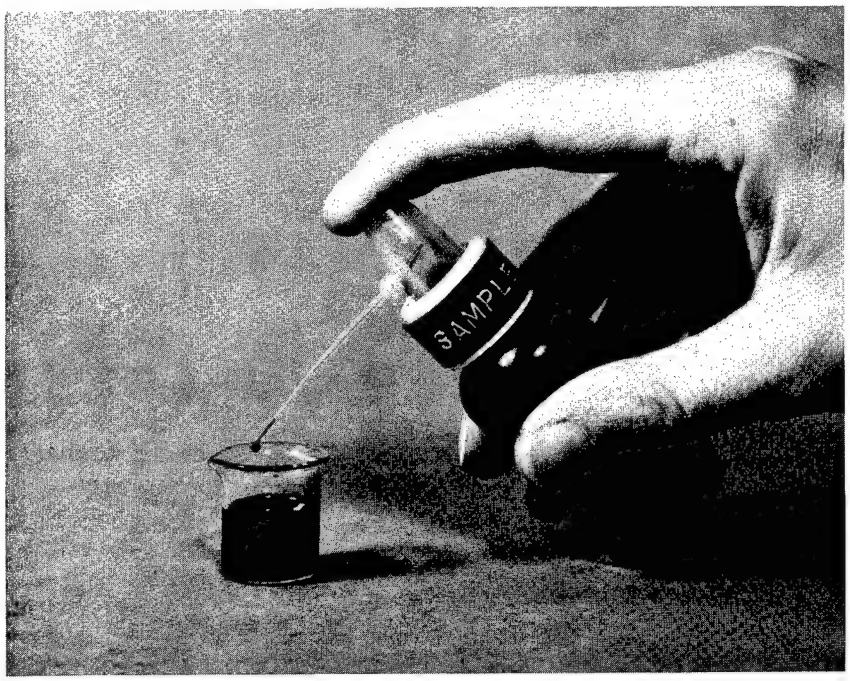

Figure 2. A Sanz-Spinco pipette in use.

of an acceptable micro colorimeter or spectrophotometer. Both the Beckman/ Spinco* spectro-colorimeter and the Coleman** Model 6C spectrophotometer equipped with an ultramicro cuvette have been employed. Each has advantages and disadvantages, though there is little or no difference as far as wave length selection is concerned, or in the ease with which the analytical cell is filled and emptied. Both instruments have proven satisfactory for ultramicro use.

The read-out dial on the Spinco instrument is a volt meter and the reading of absorbance can be made from a calibrated dial. This is considered to be more satisfactory and convenient than the galvonometer-type read-out provided by the Coleman equipment.

The test cell in the Spinco instrument is 0.64 cm in depth (light path, 0.64 cm), whereas the Coleman gear provides a light path of one cm. In some instances, the longer light path offers advantages. Both instruments require a solution volume of 0.1 ml for adequate filling of the cuvette.

4. Ultramicro Centrifuge

The Spinco and Coleman ultramicro centrifuges are fairly similar. With such equipment the separation of clot from serum or the separation of precipitates in the analytical procedures is more efficient than is the case with macro centrifugation. Also, the ultramicro centrifuges seldom require more than a maximum running time of 30 seconds.

5. Other Instruments

Other instruments evaluated for use in ultramicro analyses have proven quite satisfactory. For determination of pH, p CO_2, and allied values, the Radiometer*** micro development for the Astrup (3) analysis has given adequate performance. Also tested and found to be satisfactory for their particular use are the Cotlove Chloridometer+, the Baird flame photometer++, the

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*The Spinco Division of Beckman Instruments, Inc., Palo Alto, California.
**Coleman Instruments, Maywood, Illinois
***Type AMElc Astrup Micro Equipment, Radiometer, 72 Emdrupvej, Copenhagen N11, Denmark.
+Cotlove Chloridometer, Laboratory Glass and Instrument Corp., New York 31, New York.
++Baird Flame Photometer, Model KY-1, Baird-Atomic, Inc., Cambridge 38, Massachusetts.

Cahn electrobalance* and, for electrophoresis evaluation, the Photovolt densitometer**.

Enzyme rate determinations will be done on the Beckman DK-1A spectrophotometer*** using an ultramicro adaptation. Though the various methods have not been fully evaluated, indications are that the instrument will be entirely satisfactory for the analysis.

6. General

Experience to date has indicated that mere scaling down of a determination described on a macro scale to the ultramicro scale is frequently fraught with difficulties. In addition, some ultramicro procedures described in the literature for various determinations have been tried and found wanting. As a consequence, it has been necessary in some cases to modify the analytical procedures considerably to obtain acceptable results. In a few instances, it has been necessary to develop fully a satisfactory ultramicro approach to analysis.

SUMMARY

The rationale behind clinical chemical evaluation as an approach to the assessment of damage caused by a variety of experimental variables including ionizing radiation is considered, and the necessity of conducting the analyses on an ultramicro basis has been pointed out. The general concepts guiding the choice of a test battery and the selection of instrumentation are discussed with sufficient evidence accumulated to demonstrate the feasibility of application of the chosen analytical system.

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*Cahn Electrobalance, Model M-10, Cahn Instrument Company, Paramount, California.

**Photovolt Electronic Densitometer, Model 501-A with integrator and recorder, Photovolt Corporation, New York 16, New York.

***Beckman DK-1A Ratio Recording Spectrophotometer, Beckman Instruments, Inc., Fullerton, California.

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3. Astrup, P., Jorgensen, K., Anderson, O. S., and Engel, K.: The acid-base metabolism. A new approach. Lancet 1: 1035-1039, 1960.

II. PROCEDURES FOR THE DETERMINATION OF SERUM BILIRUBIN

INTRODUCTION

The diazo reaction for the determination of bilirubin as originally described by van den Berg (1) has undergone numerous modifications during the time since his publications. Among those most commonly employed have been the procedures of Malloy and Evelyn (2), Powell (3), or King and Coxon (4). The Malloy and Evelyn (2) method for total bilirubin accomplishes diazo coupling in a medium of aqueous methanol. When the reaction is carried out in an aqueous medium without methanol, a value is obtained which is designated "direct" bilirubin. The separation of unconjugated bilirubin, bilirubin monoglucuronide and bilirubin diglucuronide from serum by Billing, Cole, and Lathe (5) furnished the information necessary for interpretation of the Malloy-Evelyn procedure. Measurement of "direct-reacting" bilirubin gives an approximation of the conjugated form. Total bilirubin determination estimates both conjugated and unconjugated bilirubin. Micro modifications of the Malloy-Evelyn method have been described by Hogg and Mietes (6), Natelson (7), and O'Brien and Ibbott (8).

A new dimension was added to bilirubin analysis with the introduction of spectrophotometric procedures by Shinowara (9) and by Abelson and Boggs (10). White, Haidar, and Reinhold (11), developed a micro adaptation of this analytical approach. The spectrophotometric analysis of bilirubin as described by these authors determines only total bilirubin. Eberlein's (12) extension of this method utilizes the differential solubility in ethyl acetate and an aqueous buffer to accomplish the separate analysis of unconjugated bilirubin, which is usually designated "free" bilirubin.

It was considered necessary to compare these two different methods of bilirubin analysis on an ultramicro basis in order to have an adequate evaluation of the results obtained by the spectrophotometric method as compared to the older, more widely used diazo procedure.

PROCEDURES

1. Preparation of Standards

Best results can be obtained with a standard which meets the criteria of maximum purity, stability, practicability of preparation, and reproducibility. The method of purification of bilirubin described by Henry, Jacobs, and Chiamori (13) makes possible the preparation of a bilirubin of constant purity as judged by the attainment of maximum extinction coefficient. Using this method, commercial bilirubin was purified until an extinction coefficient of 59,000 or higher at 453 mμ in chloroform was obtained.

Recent emphasis has been on the preparation of a bilirubin standard in aqueous protein solution (9, 14, 15). This type of standard is to be preferred, inasmuch as the analysis is accomplished in a similar medium, and also because the wave length of maximum absorption of bilirubin in an aqueous protein solution differs from that in a chloroform, methanol solution after diazo coupling (16). These considerations led to the preparation of standards as follows:

a. Weigh 10 mg of pure bilirubin into a 50 ml beaker. Use a semi-micro balance.

b. Add 2 ml of one per cent aqueous solution of sodium carbonate. Heat gently on a hot water or steam bath until the bilirubin goes into solution.

c. Add 20 ml of albumisol and mix thoroughly with a small stirring rod. Albumisol is a five per cent buffered solution of human serum albumin (Merck, Sharpe, and Dohme).

d. Transfer quantitatively to a 100 ml volumetric flask. Rinse the beaker with several small portions of albumisol and transfer each to the flask. Make up to 100 ml with albumisol and mix. The standard is equivalent to 10 mg bilirubin per 100 ml. The preparation of the standard should be performed in a dimly lighted room. The solution may then be subdivided into conveniently sized aliquots and stored frozen. The frozen standard is stable for at least one month.

2. Analytical Methods

a. DIAZO REACTION, ULTRAMICRO, FOR BILIRUBIN DETERMINATION

Adapted from Lathe and Ruthven: J. Clin. Pathol. 11: 155 (1960).

1) Reagents:

Absolute methyl alcohol (C. P. or reagent grade). To two liters methanol in a round bottom flask add two gm 2,4-dinitrophenylhydrazine and 0.5 ml conc. H_2SO_4. Distill under anhydrous conditions. Discard the first one-third of the distillate. Collect only the middle one-third. Discard the last one-third (residue in flask).

Diazo reagent, solution "A". Dissolve one gm of reagent grade sulphanilic acid in 0.83 ml concentrated hydrochloric acid and dilute to 100 ml with water.

Diazo reagent, solution "B". Dissolve 0.25 gm of reagent grade sodium nitrite in distilled water and dilute to 50 ml. This solution should be prepared fresh weekly.

Mixed diazo reagent. Mix ten ml of diazo "A" solution with 0.3 ml of diazo "B" solution. Use within 10-20 minutes after mixing.

2) Procedure:

Add reagents in the order described below to a 400 µl polyethylene centrifuge tube, permitting one minute to elapse between the addition of the diazo reagents and the methanol; mix; allow to stand for five minutes.*

		Total		Direct	
		Blank	Unknown	Blank	Unknown
1.	Serum	20 µl	20 µl	20 µl	20 µl
2.	H_2O	70 µl	70 µl	70 µl	70 µl
3.	Mixed diazo		60 µl		60 µl
4.	Diazo "A"	60 µl		60 µl	
5.	Methanol	150 µl	150 µl		
6.	H_2O			150 µl	150 µl

*The mixing procedure for the 400 µl reaction tubes has been developed with the Vortex Junior mixer. Each analyst can work out this technique without difficulty by practice. By holding the tube at an angle against the edge of the cup of the mixer (see Figure 1), thorough mixing can be accomplished very quickly.

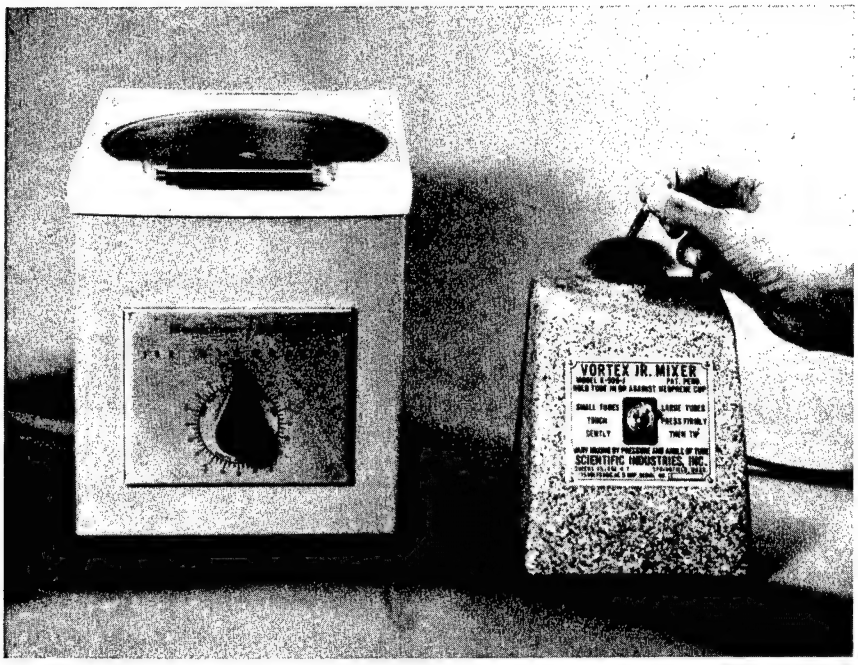

Figure 1. The micro centrifuge (microfuge) and Vortex Junior in use for mixing the contents of a 400 µl polyethylene tube are illustrated.

Read absorbance (A) of sample and blank using water as reference at wave length 540 mμ on the Coleman Junior spectrophotometer with the ultramicro cuvette.

3) Calculation:

A (unknown) - A (blank) x factor = mg per 100 ml

b. SPECTROPHOTOMETRIC PROCEDURE, ULTRAMICRO, FOR TOTAL BILIRUBIN DETERMINATION

Adapted from White, Haidar, and Reinhold: Clin. Chem. 4: 211 (1958).

1) Reagents:

Buffer (phosphate pH 7.4). Dissolve 1.74 gm of potassium dihydrogen phosphate (KH_2PO_4) and 7.65 gm of disodium hydrogen phosphate heptahydrate ($Na_2HPO_4 . 7H_2O$) in distilled water and make up to one liter. Adjust the pH to 7.4 on a pH meter.

2) Procedure:

To a 400 μl test tube add 200 μl of phosphate buffer, pH 7.4. Pipette 20 μl of serum or standard into the buffer and mix thoroughly by agitation with the Vortex mixer. Centrifuge to remove the foam at the surface and determine absorbance (A) at 460 mμ and 575 mμ, against a buffer blank on the Spinco spectro-colorimeter.*

3) Calculation:

A at 460 - A at 575 x factor = bilirubin, mg per 100 ml

c. SPECTROPHOTOMETRIC PROCEDURE, ULTRAMICRO, FOR FREE BILIRUBIN DETERMINATION

Adapted from Eberlein: Pediatrics 25: 878 (1960).

1) Reagents:

Phosphate buffer (pH 5.0). Dissolve 27.2 gm of potassium dihydrogen phosphate (KH_2PO_4) in distilled water and make up to one liter. Adjust the pH to 5.0 with 5 normal or stronger sodium hydroxide.

---

*By sucking the sample back into the pipette from the cuvette and recentrifuging it, one sample can be read at both wave lengths. Alternatively, duplicate samples may be prepared initially.

Ethyl acetate (reagent grade).

2) Procedure:

To a 400 µl test tube add 80 µl of phosphate buffer, 20 µl of serum or standard, and 200 µl of ethyl acetate, in that order.

Mix thoroughly (Vortex mixer), centrifuge for 30 seconds, then mix and centrifuge again.

Determine absorbance (A) of the ethyl acetate layer at 460 mµ and 575 mµ* read against an ethyl acetate blank, on the Spinco spectro-colorimeter.

3) Calculation:

A at 460 - A at 575 x factor = free bilirubin, mg per 100 ml

RESULTS

1. Standardization. The Conversion Factor.

The establishment of a standard curve and the determination of a conversion factor are illustrated in Figure 2. In this experiment, the diazo method was used and the final color was read in the Coleman Junior spectrophotometer (ultramicro) at 540 mµ. From a plotted curve obtained with standards representing bilirubin concentrations of 1, 2, 3, and 4 mg per 100 ml, a conversion factor was determined. The factor must be determined for each individual instrument.

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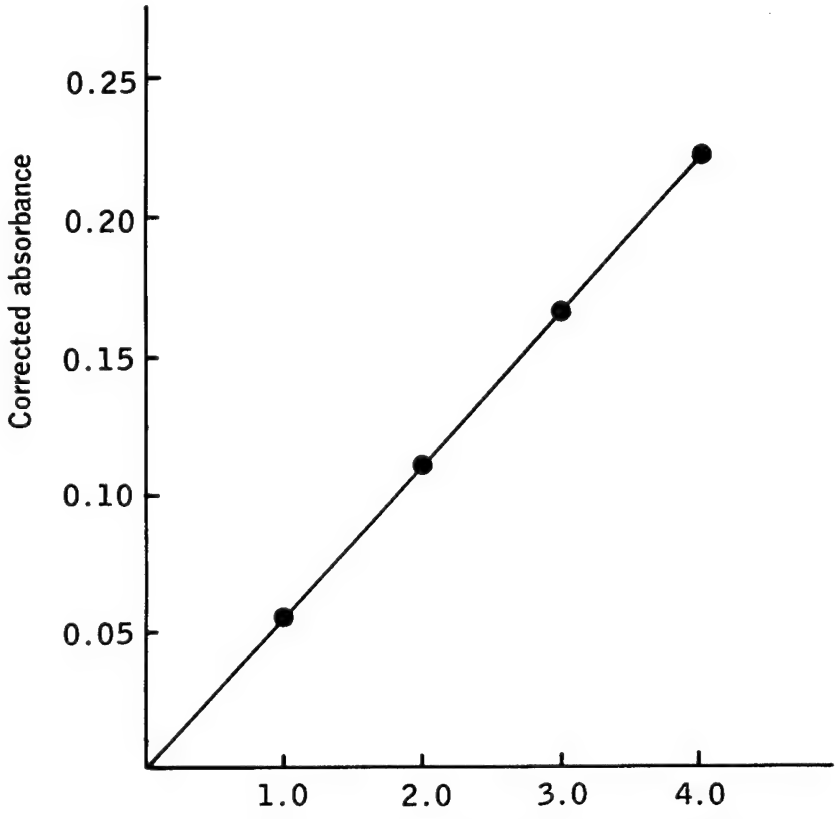
*Both White, et al. and Eberlein used a Beckman DU or Beckman B spectrophotometer and wave lengths of 455 and 575 mµ. The wave lengths were chosen because at these points oxyhemoglobin gives almost identical absorbance. Therefore, the absorbance at 455 mµ minus the absorbance at 575 mµ corrects for the amount of hemoglobin present. The Spinco spectro-colorimeter did not give sufficiently precise resolution to agree with the Beckman B. This wave length correction should be determined for each microspectrophotometer. Spectral waves were determined on the Spinco instrument using two hemoglobin concentrations and a bilirubin standard. The main absorption band of bilirubin was rather broad and, over the range 465 to 455 mµ, the increase was relatively insignificant. At wave lengths of 460 and 575 mµ the absorbance of the hemoglobin was the same. Therefore, the wave lengths of 460 and 575 mµ were chosen.

Fig. 2. Establishment of a conversion factor for the determination of bilirubin by the ultramicro diazo reaction.



$$\text{Factor} = \frac{\text{Bilirubin concentration}}{\text{Absorbance}}$$

Bilirubin concentration mg per 100 ml	Corrected absorbance	Factor
1	0.055	$\frac{1}{0.055} = 18.2$
2	0.110	$\frac{2}{0.110} = 18.2$
3	0.165	$\frac{3}{0.165} = 18.2$
4	0.220	$\frac{4}{0.220} = 18.2$
		Mean: = 18.2

The linearity of the reaction having been demonstrated (Figure 2), the use of a conversion factor is permissible. The reliability of a conversion factor was checked further by modifying the White, et al. (11) and Eberlein (12) methods to a macro scale. These procedures utilized 0.5 ml of serum in 4.0 ml of buffer, pH 7.4, for total bilirubin, or 0.5 ml serum in 2.0 ml buffer, pH 5.0, followed by 4.0 ml ethyl acetate for the extraction of free bilirubin. The readings were taken on a Beckman B spectrophotometer.

2. Reproducibility of Standards

Table 1 presents data showing the reproducibility of the preparation of standard solutions and the close agreement of the conversion factors for the diazo method at varying bilirubin concentrations. The reliability of the conversion factor determination for the ultramicro and macro spectrophotometric determinations is shown in Table 2. Reproducibility is further illustrated in Table 3, in which a comparison of standards prepared with different types of protein solution is made. It is evident not only that standard preparations may be readily duplicated, but also that several types of protein solution are acceptable media for their preparation.

3. Hemoglobin Effects

One of the advantages of the spectrophotometric analysis is that it corrects for hemoglobin when it is present in the serum. In contrast, the presence of hemoglobin interferes with the diazo reaction and the results obtained are erroneously low. A comparison was made of the two methods by use of a standard to which known and varying amounts of hemoglobin were added, and the results are recorded in Table 4. The interfering effect of hemoglobin is demonstrated, and the results serve to emphasize the advantage of the spectrophotometric method in its presence.

4. Comparison of Methods

Finally, the two methods were compared on specimens of serum. Table 5 records the results obtained with serum from infants and the results using serum from adults are listed in Table 6.

Table 1

Reproducibility of Standard Preparations Measured by the Ultramicro Diazo Reaction*

Bilirubin content of standard (mg/100 ml)	Total bilirubin Corrected absorbance**	
	Standard 1	Standard 2
1	0.055 (18.2)***	0.055 (18.2)
2	0.110 (18.2)	0.110 (18.2)
3	0.165 (18.2)	0.170 (17.64)
4	0.220 (18.2)	0.220 (18.2)

*Standards prepared by dissolving bilirubin in albumisol. Final color measured on Coleman Junior spectrophotometer with ultramicro cell.

**It was necessary to determine the amount of color (absorbance) produced by the protein diluting solution, alone. This value was deducted from the absorbance of each bilirubin standard, and the resulting value is the corrected absorbance (see also Figure 1).

***Numbers in parentheses are conversion factors (see also Figure 1).

Table 2

Determination of Conversion Factors for the Spectrophotometric Procedures

Bilirubin content of standard (mg/100 ml)	Corrected absorbance*		
	Total bilirubin	Free bilirubin	
	Micro	Micro	Macro
1	0.034 (29.4)	-	0.09 (10.1)
2	0.070 (28.6)	0.075 (26.7)	0.187 (10.7)
3	0.105 (28.6)	0.113 (26.5)	0.278 (10.8)
4	0.140 (28.6)	0.152 (26.3)	0.365 (10.9)

*Microanalysis done with Spinco spectro-colorimeter; macroanalysis done with Beckman B spectrophotometer.

Table 3

Comparison of Total Bilirubin Standards
Standardization Made Using Different Types of Protein Preparations

Bilirubin content of standard (mg/100 ml)	Corrected absorbance*		
	1	2	3
1	0.083	0.085	0.085
2	0.168	0.169	0.167
4	0.332	0.339	0.340

*Standard 1 prepared with human serum; 2 with citrated human plasma; 3, with albumisol. Macro spectrophotometric procedure; readings with Beckman Model B.

Table 4

The Effect of Hemoglobin on the Determination of Bilirubin

Hemoglobin concentration (mg/100 ml)	Bilirubin concentration determined mg/100 ml		
	Spectrophotometric*	Diazo*	Malloy-Evelyn (micro)
0	2.0	2.0	2.0
100	1.9	1.7	1.6
200	2.0	1.8	1.6
300	2.0	1.7	1.5
400	2.0	1.8	1.7
500	2.03	1.7	1.6
800	2.03	1.8	1.7

*The procedures described in the text were used for these determinations.

Table 5

The Ultramicro Diazo Method Compared to the Spectrophotometric Method Using Serum Obtained from Human Infants

Diazo method Bilirubin, mg/100 ml		Spectrophotometric method Bilirubin, mg/100 ml	
Total	Indirect*	Total	Free
1.8		1.8	
1.8		1.8	
19.6	15.6	22.2**	16.5
18.0	14.0	17.7	11.4
17.4	13.5	16.4	10.7
11.3		11.7	
4.5		4.0	
9.5		9.0	
12.4		11.3	
14.6		12.6	
19.4		25.0**	

*This value was obtained by deducting the value for "direct-reacting" from that of total bilirubin.

**Marked hemolysis.

Table 6

A Comparison of Total Bilirubin Determined by the Ultramicro and Macro Diazo Methods to the Spectrophotometric Method Using Serum from Adult Human Subjects

	Bilirubin, mg/100 ml		
Specimen	Diazo method ultramicro	Diazo method macro*	Spectrophotometric method, macro
1	0.82	0.76	1.23
2	0.36	0.22	0.77
3	0.46	0.42	1.05
4	9.5	10.0	8.0
5	4.1		4.5
6	5.4	6.0	5.0
7	7.9		7.7
8		24.8	25.0
9		49.0	46.0

*Method of Lathe and Ruthven (17).

DISCUSSION

1. The Bilirubin Standard

The ease of preparation and reproducibility of the standard has been demonstrated. It should be noted, however, that not all protein preparations are satisfactory for this purpose. One bottle of albumisol and one of plasminate (five per cent protein solution containing albumin and globulin, Cutter) gave low readings with the diazo reaction, possibly because of the presence of some substance which inhibited the reaction. The stability of standards in the frozen state allows the procedure to be checked daily for a period of one month with the same standard.

2. Reaction Time

The time allowed for the development of color in the aqueous diazo procedure follows the suggestion of Lathe and Ruthven (17). They demonstrated that little was to be gained by allowing the reaction to proceed for 30 minutes provided the pH and reagent concentrations were maintained as described in their suggested method. Their reaction time, which has been adopted here, utilizes the color development at five minutes as an estimate of the amount of conjugated bilirubin. Their data indicate that this provides for the coupling of "as little of the bilirubin and as much of the conjugated bilirubin as possible." This seems to be a fair compromise, inasmuch as conditions have not yet been described which accomplish coupling of all of the conjugated bilirubin to the exclusion of all of the unconjugated bilirubin. The same conclusions are valid for the aqueous methanolic reaction mixture (total bilirubin).

3. Spectrophotometric Analysis

The advantages of the spectrophotometric procedure are that only one reagent is required for total bilirubin determination and that reaction time is not a consideration. Standardization with pure bilirubin in aqueous protein solution permits the establishment of a conversion factor which takes into account the extinction coefficient of bilirubin in the presence of protein. Furthermore, hemoglobin, which is often present in serum, does not interfere with the accurate determination of bilirubin as it does in the diazo reaction (Table 4).

This spectrophotometric method has been criticized for use with adult serum because of the errors possibly introduced by the presence of

carotene as well as other lipochrome pigments. The results recorded in Table 6 indicate that, within the normal range, the results are indeed higher than those obtained with the diazo reaction. However, when the bilirubin was elevated, there was no consistent trend in the differences obtained with the two methods. A further attempt was made to check this point by addition of an alcoholic solution of carotene to a bilirubin standard of known concentration. After thorough mixing, this was analyzed by the method of Bessey, et al. (18) and found to have a concentration of 1.0 mg carotene per 100 ml. Spectrophotometric analysis of this solution gave a bilirubin concentration only 0.1 mg per 100 ml higher than the known quantity. These data seem to rule out the presence of carotene as a major contraindication to the use of the spectrophotometric procedure.

Table 5 shows the comparative results obtained by the two methods. Comparison of results for free bilirubin with indirect bilirubin in the diazo coupling reaction shows only a fair agreement. The conditions for measuring direct bilirubin are arbitrary at best, and better agreement could hardly be expected. It is likely that the solvent partition method gives an estimate of free and conjugated bilirubin which is more nearly exact than that obtained from the diazo reaction. Recently Tisdale and Welch (19) have compared the two results obtained by utilizing the Malloy-Evelyn method (2) to those obtained with the Eberlein solvent partition method. Their results were consistently and significantly higher by the diazo reaction. They also stated inability to determine a consistent conversion factor for the spectrophotometric method, either for the ethyl acetate extract or the aqueous phase. If one compares the micro modification of the Lathe and Ruthven (17) diazo reaction for total bilirubin as described herein with the spectrophotometric procedure for total bilirubin of White, et al. (11), such large discrepancies do not occur (Table 6). Furthermore, the determination of a conversion factor for free bilirubin extracted from a pH 5.0 phosphate buffer according to Eberlein (12) was quite reproducible (Table 2). The method described previously, utilizing an extract made by mixing and centrifuging twice, gives satisfactory, reproducible separation. The analyst must be aware of the possibility of separation of water from the ethyl acetate when the extract, warmed by centrifugation, is transferred to the cooler cuvette. Clouding, so caused, can be eliminated by addition to the extract of a small amount of anhydrous sodium sulfate.

The lack of sufficient information to justify the application of data obtained from attempts to separate the mono- and diglucuronides (19 - 22) seems to contraindicate application of these procedures at this time.

4. Reproducibility

Finally, the data herein reported demonstrate a high degree of reproducibility both for the diazo reaction and for the spectrophotometric procedure, free and total. Whereas it is recognized that neither method has the virtue of complete accuracy, the precision under carefully standardized conditions indicates the usefulness of either one as a means of following changes in bile pigment metabolism.

SUMMARY AND CONCLUSIONS

Ultramicro methods utilizing both the van den Berg (1) reaction and spectrophotometric analysis for the determination of total bilirubin and of "direct-reacting" and "free" (unconjugated) bilirubin have been compared. Whereas neither method gives absolutely accurate results, the precision attained by both is sufficiently good to justify the use of either one to establish the existence of an abnormality in bilirubin metabolism and to follow its progress.

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